# Cloning of Putative Cobalamin Reductases of *Thermosipho melanesiensis*

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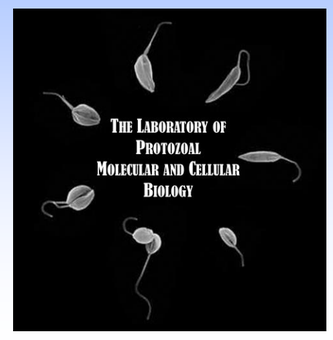


## Introduction

Cobalamin, commonly known as Vitamin $B_{12}$, is a vitamin that plays an essential role in keeping human nerve and blood cells healthy. It is also a cofactor for the synthesis of enzymes involved in citric acid cycle metabolism, DNA synthesis, and gene regulation.(1)

Only certain Bacteria and Archaea possess the required enzymes for Cobalamin biosynthesis. Eukaryotes cannot synthesize Cobalamin *de novo*, but obtain it in one of two ways: via gut microorganisms that synthesize Cobalamin, or via food sources. Humans use the latter method by consuming animal products.(2,3)

Our aim is to uncover the unknown gene identities of three reductase enzymes (Figure 1) in *Thermosipho melanesiensis* that are suspected to be required for *de novo* Cobalamin synthesis. Previous research on protein comparison to *Salmonella enterica* has targeted three DNA sequences as possible reductase genes.(4)

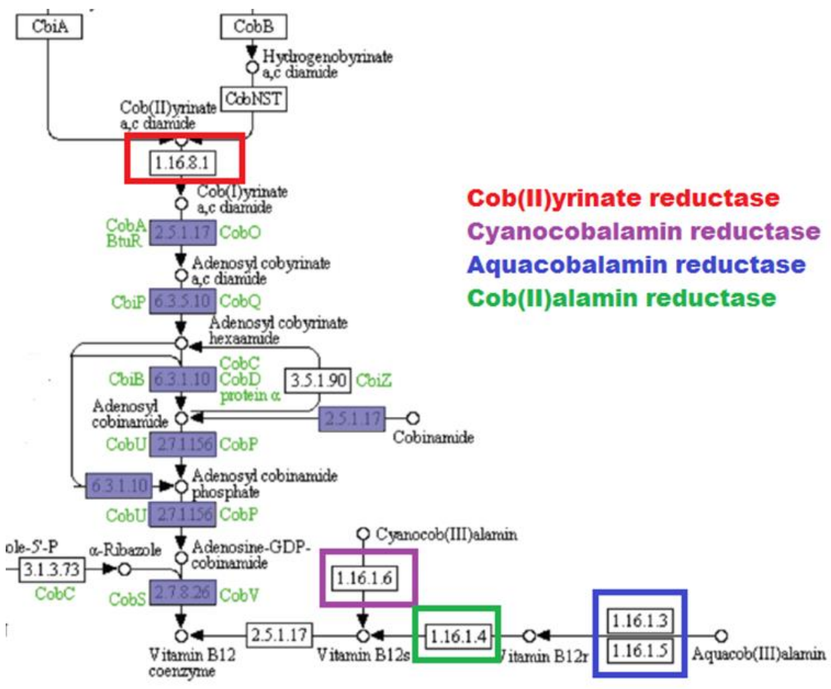


**Fig. 1. $B_{12}$ biosynthesis and recycling in *Thermosipho melanesiensis*** (KEGG). The missing reductases are displayed within colored boxes. Red is Cob(II)yrinate reductase (1.16.8.1), Purple is cyanocobalamin reductase (1.16.1.4), Blue is aquacobalamin reductase (1.16.1.3 and 1.16.1).

## Methods

**Subcloning *T. melanesiensis* Putative Cobalamin Reductase Gene Insert Into Expression Vector**

Clones that contained correctly sequencing gene inserts and the expression vector pTrcHis-B (ThermoFisher Scientific) were digested by the restriction endonucleases *Xho*I and *Kpn*I-HF (New England Biolabs) as follows in 30 ul reactions: 5 ul insert or vector, 3 ul 10X cutsmart buffer, 19 ul water, 1.5 µl *Xho*I, and 1.5 µl *Kpn*I. Digests were incubated for 5 hours at 37 $^{o}$C. Digests were analyzed by agarose gel electrophoresis as described above.

Ligations of inserts and pTrcHis-B vector were set up in 30 µl reactions in the following ratios of vector to insert: 1:0, 1:3, and 1:5. Ligations incubated overnight at 16 $^{o}$C and used to transform One Shot$^{tm}$ TOP10 chemically competent *E. coli* and plated on LB agar containing 100 µg/ml ampicillin. Ten colonies from each insert were analyzed by colony PCR and restriction digestion analysis.

**Nickel Affinity Chromatography**

Expression vector clones verified by colony PCR and restriction digestion analysis were cultivated in 50 ml LB containing 100 µg/ml ampicillin and 1 µM IPTG to induce expression of insert. Cells were pelleted at 4,000 rpm 4 $^{o}$C for 10 minutes. Cells were resuspended in 1 ml binding buffer (50 mM $NaH_2PO_4$ @ pH 7.5, 200 mM NaCl, 10 mM Imidazole; ClaremontBio Solutions). Lysozyme solution (ThermoFisher Scientific) was added to suspension at a concentration of 50 µg/ml. Incubated for 30 minutes at room temperature. Lysis was conducted using an ultrasonicator. Cell lysate was passed through a HisExpress column (ClaremontBio Solutions). 3 ml of wash buffer (50 mM $NaH_2PO_4$ @ pH 7.5, 300 mM NaCl, 20 mM Imidazole; ClaremontBio Solutions) were passed through the column and collected in 1 ml aliquots. This was followed by 2 ml of elution buffer (50 mM $NaH_2PO_4$ @ pH 7.5, 200 mM NaCl, 250 mM Imidazole; ClaremontBio Solutions) and collected as 1 ml aliquots.

**SDS-PAGE Analysis**

Purified protein samples were analyzed by SDS-PAGE as follows: 20% or 12.5% resolving gels with 4% stacking gels were loaded with 40 ul of the following in order: Crude lysate, wash 1, wash 2, wash 3, elution 1, and elution 2. Gel were run at 37 mA per gel for 1 hour and 15 minutes. Gels were stained using SYPRO Ruby protein stain (Bio-Rad) overnight, washed with 10% methanol then water and then imaged.

## Results

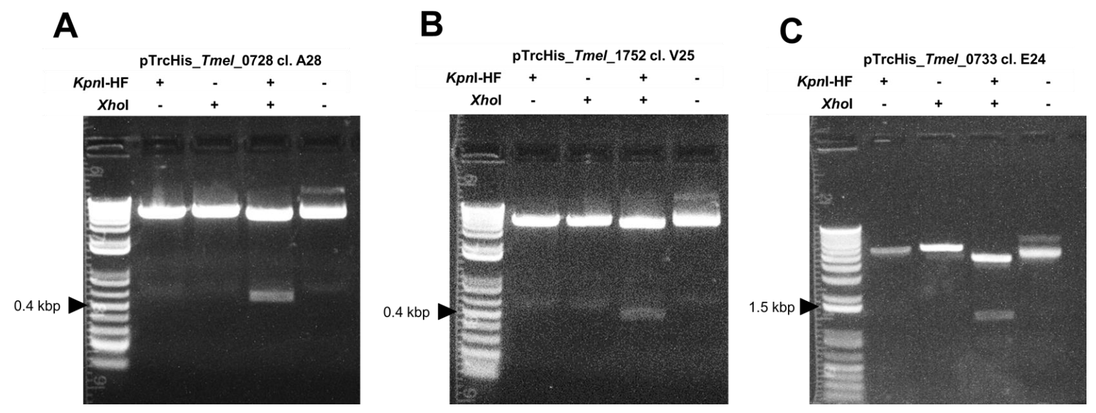


**Figure 2: Insert Verification by Restriction Digestion Analysis.** 0.8% agarose gel electrophoresis. Lane 1, 1kb plus DNA ladder (0.4 kbp and 1.5 kbp shown), Lane 2, Kpn-HF, Lane 3, XhoI, Lane 4, both Kpn-HF and XhoI, Lane 5, control. (A) pTrcHis_*Tmel*_0728 cl.28, (B) pTrcHis_*Tmel*_1752, (C) pTrcHis_*Tmel*_0733

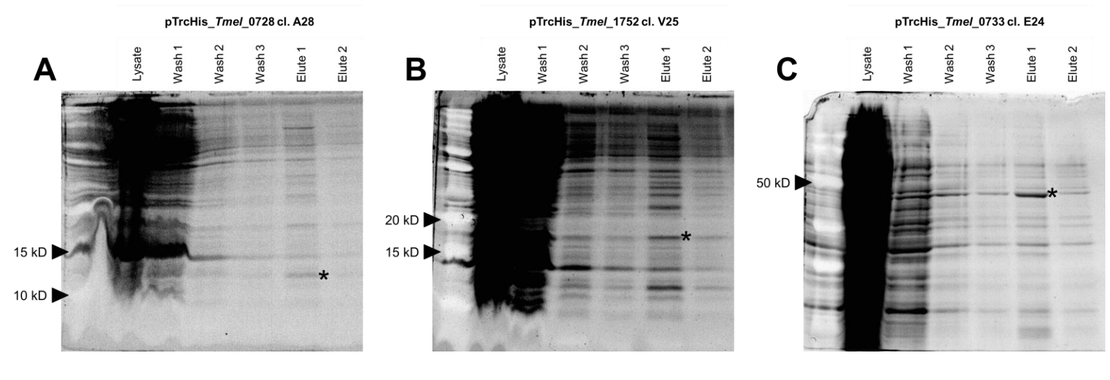


**Figure 3: SDS-PAGE Analysis of $Ni^{2+}$ Affinity Chromatography of His-tagged Proteins.** (A) 6His-Tmel_0728 expected molecular weight of 13.5 kD indicated by asterisk, 15 kD and 10 kD marker shown. (B) 6His-Tmel_1752 expected molecular weight 17.2 kD indicated by asterisk. (C) 6His-Tmel_0733 expected molecular weight 48.2 kD indicated by asterisk.

## Conclusions

The putative genes (Tmel_0728, Tmel_1752, and Tmel_0733) were successfully cloned. The genes were successfully inserted into the expression vectors, and resolved into bands at their expected sizes (Figure 2). After inducing expression, the genes produced proteins of expected molecular weights (Figure 3).

## Future Works

The proteins produced from the expression of the putative cobalamin reductase genes will undergo biochemical analysis to confirm that their function is indeed involved in the Cobalamin (Vitamin $B_{12}$) biosynthetic pathway.

## Bibliography

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